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The importance of individual nucleotides for the structure and function of rRNA molecules in *E. coli*

A mutagenesis study

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Methods of in vitro mutagenesis were employed to determine the importance of individual nucleotides within the ribosomal RNAs for the structure and function of *E. coli* ribosomes. A series of defined nucleotides in the genes for the 5 S and 16 S RNA were altered by transition and transversion mutations using either oligonucleotide-directed or bisulfite-catalyzed mutation procedures. Plasmids harbouring the mutated rRNA genes were expressed and the ribosomes containing such altered RNAs were investigated for impairments in RNA-protein interaction assembly and mRNA-coded tRNA binding.

ribosomal RNA *Mutagenesis* *Protein-RNA interaction* *tRNA binding* *Subunit association*

1. INTRODUCTION

A number of recent reports have convincingly demonstrated that some RNAs have enzyme-like functional activities [1,3]. Similar observations are true for ribosomal RNAs, pointing to a functional importance of defined sequence regions for individual steps in the mechanism of translation [3–6].

Using methods of chemical modification, cross-linking and limited enzymatic hydrolysis, we and others have provided a catalogue of individual nucleotides within the ribosomal RNAs that may be of potential importance for a number of ribosomal functions [5–11]. Whether these nucleotides are actually important and how they mediate possible functions can be determined by base substitutions of the corresponding sites and assessment of the structural and functional impairments of the resulting molecules.

Here we report on the analysis of effects caused by single- and multiple-site mutations within the gene for the ribosomal 5 S RNA, and on the construction and functional analysis of point mutants at the 3′-end of the 16 S RNA. The mutated 5 S RNAs were analyzed for their capacity to form stable complexes with the binding proteins L5, L18 and L25. Furthermore, the effects on the tRNA binding capacity of ribosomes containing such mutated 5 S RNAs were investigated. The same assay was employed for ribosomal mutants with base changes at the 3′-end of the 16 S RNA. These mutants were additionally tested with respect to their subunit association equilibrium. It turned out that a single-site mutation in the 16 S RNA decreased the stability of 70 S ribosomes dramatically.

2. MATERIALS AND METHODS

The following strains were used for the construction and expression of the rRNA mutants: HB101

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[12], JM103/JM105 [13], CSR603 [14] and WM1151, a derivative of W3110 [15]. The ribosomal RNAs containing plasmid vectors pKK3535 [16] and pKK223-3 [17] were used for cloning and expression of mutant genes. The expression vector pLSK34-1, a pK-01 derivative [18], was kindly provided by R. Kölling and used for expression of mutant 5 S RNA genes. The M13 phage DNA mp8, mp10 were purchased from P.L. Biochemicals. Methods for recombination of DNA were applied as described by Maniatis et al. [19]. DNA sequencing was performed according to Sanger et al. [20]. For the selective expression of plasmid-coded rRNAs in maxicells the method developed by Stark et al. [21] was followed. Oligonucleotide-directed point mutants in the 5 S RNA gene were constructed as described [22,23]. The bisulfite-catalyzed transition mutants were constructed according to [24]. Details of the mutation procedures of the 5 S RNA and 16 S RNA mutants are described elsewhere [25,26].

3. RESULTS AND DISCUSSION

The following ribosomal RNA mutants have been obtained: (i) Point mutations constructed by oligonucleotide-directed mutagenesis at the 5 S RNA positions: 41 (G to C); 66 (A to C) and 103 (U to G). (ii) Bisulfite-catalyzed transition mutants within the 5 S RNA. A collection of G to A transitions that have been sequenced is shown in table 1. (iii) Oligonucleotide-directed point mutants at the 3′-end of the 16 S RNA: C_{1399} to A/G_{1401} to C; C_{1399} to A/G_{1401} to U and G_{1416} to U.

The different mutants were screened by dot-blot analysis [27] and in the case of the bisulfite-catalyzed transition mutants by two-lane sequencing. In all cases the base changes were verified by direct DNA sequencing [20]. An example for one of the 16 S RNA mutants is shown in fig.1. Most of the 5 S RNA mutants were additionally characterized by RNA sequencing of the mutant genes, after expression in maxicells.

Table 1

Collection of bisulfite-catalyzed G to A transition mutants

```
                  10        20        30        40        50        60        70        80        90       100       110
          5' TGCCTGGCGGCAGTAGCGCGGTGGTCCCACCTGACCCCATGCCGAACTCAGAAGTGAAACGCCGTAGCGCCGATGGTAGTGTGGGGTCTCCCCATGCGAGAGTAGGGAACTGCCAGGCAT 3'

MUTANTS
15-02     ..........|..........|..........|..........|..........|.....A....|..........|..........|..........|..........|..........|..........
15-23     ..........|..........|..........|..........|..........|..........|..........|.....A....|..........|..........|..........|..........
15-16     ..........|..........|..........|..........|..........|..........|..........|..........|..........|.....A....|..........|..........
15-04     ..........|..........|..........|..........|..........|.....A....|..........|..........|..........|..........|..........|..........
15-05     ..........|..........|..........|..........|..........|.....A...A|..........|..........|..........|..........|..........|..........
15-08     ..........|..........|..........|..........|..........|..........|......A...|..........|..........|.......A..|..........|..........
15-10     ..........|..........|..........|...A......|..........|..........|..........|..........|..........|.........A|..........|..........
15-07     .....A....|..........|..........|..........|..........|..........|.....A....|..........|.....A....|.......A..|..........|..........
60-48     .....A....|..........|..........|..........|..........|..........|.....A....|.....A.A..|..........|..........|..........|.....A....
140-25    ..........|..........|..........|...A......|.....A....|..........|.....A....|.....A....|..........|..........|..........|..........
60-30     ..........|...A.....A|..........|..........|..........|.....A....|......A...|..........|.....A....|..........|.....A....|..........
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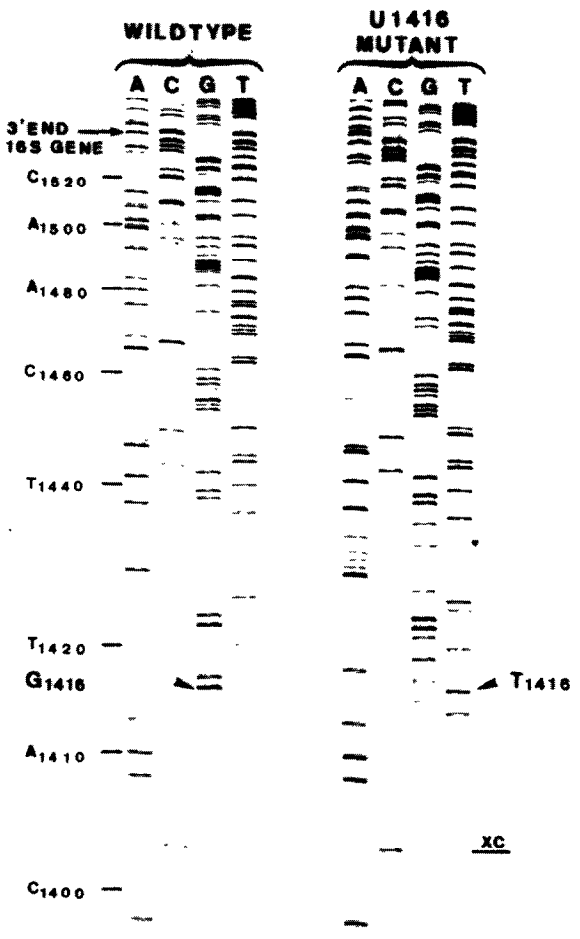

Fig.1. DNA sequencing gel demonstrating the U_{1416} base change mutation in the 16 S RNA gene. The sequences of DNA fragments comprising the 3'-end of the 16 S RNA are shown from wild-type and U_{1416} mutant molecules. The base change at position 1416 is indicated by an arrow. XC denotes the xylene cyanol marker dye.

3.1. *Expression of mutant RNAs*

The 5 S RNA genes with point mutations at positions 41, 66 and 103, as well as the 16 S RNA point mutants, were recloned in the plasmid vector pKK3535 [16] where the complete rrnB operon is expressed under the control of the normal ribosomal RNA promoters. The 5 S RNA mutant genes constructed by bisulfite-catalyzed transitions were cloned in the expression vector pLSK34-1 where transcription could be induced by the addition of IPTG to the growing cells. Both expression systems were similar in efficiency but had the disadvantage that wild-type ribosomal RNA, transcribed from the 7 chromosomal operons, contaminated the mutant RNAs. We estimate the contribution of mutated RNA to wild-type RNA to be about 50% with slight variations for the different mutants ([22], unpublished).

For a selective expression of the mutated RNAs we used the maxicell labeling procedure developed by Stark et al. [21]. Using this approach pure radioactive mutant RNAs could be obtained and also ribosomes containing mutated rRNAs could be distinguished from those assembled from wild-type RNA. Fig.2 shows an example of the selective ribosomal RNA transcription from plasmids using the maxicell procedure.

3.2. *Protein binding studies employing mutated 5 S RNAs*

The mutant 5 S RNA molecules isolated after maxicell labeling or, in the case of the bisulfite mutants, after induction of cell cultures with IPTG, were purified and renatured to obtain the active protein binding conformer [5]. The 5 S RNA mutants induced by IPTG can be separated from the chromosomal transcribed wild-type 5 S RNAs due to a difference in processing. The defective processing provides 5 S RNA molecules, two bases longer than normal 5 S RNA. The protein binding of the longer molecules is, however, not affected [28]. The ribosomal proteins L5, L18 and L25, kindly provided by Dr J. Dijk, were used for complex formation with the mutated RNAs. The 5 S RNA-protein complexes were quantified by the filter binding procedure [29] and binding constants were determined as described in [23,30]. Table 2 shows a collection of some of the apparent binding constants for the proteins L18 and L25 where helix II, the putative interacting domain for L18, has been altered by an increasing number of mutations. The theoretical stability of helix II from the various mutants, calculated according to the Zuker algorithm [31], is shown for comparison in table 2. Interestingly, the binding constants for L18 are not as strongly affected as one would expect according to the hypothesis that helix II, and especially the bulged out nucleotide A66, constitutes the main binding site for the ribosomal protein L18 [11]. In contrast, and as table 2 clearly shows, the binding of the ribosomal protein L25 seems to be more stringently related to an intact helical geometry of

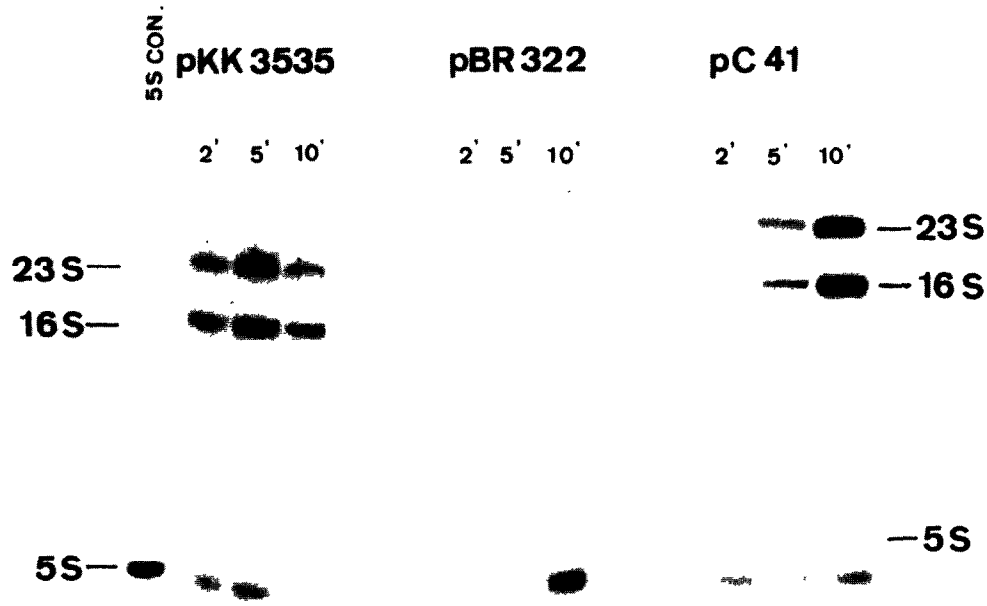

Fig.2. RNA analysis after maxicell labeling. Total RNA preparations from CSR 603 cells containing the different plasmids pKK3535, pBR322 and pC41, a pKK 3535 derivative with a point mutation at position 41 of the 5 S RNA gene, were separated after UV irradiation. The irradiation time in minutes and the positions of the ribosomal RNAs are indicated. Note that pBR322 does not code for ribosomal RNAs.

Table 2

Comparison of helix II stability and binding constants

WILD TYPE	--G-C-G-C-G-G-U-G-- --C-G A U-G-C-C-G-C--	- 42	3.1×10^5	2.9×10^6
C-66	--G-C-G-C-G-G-U-G-- --C-G-(C)-G-C-C-G-C-- U	-61.7	3.8×10^5	5.3×10^6
15.04	--G-C G-C - G-G-U-G-- --C-G A(A)C-C-G-C-- U	- 23.9	3.3×10^5	2.7×10^6
60.30	--G-C-G-C-(A)-G-U-G-- --C-(A)-A-U-G-C C-G-C--	- 9.7	2.7×10^5	2.5×10^6
140.34	-(A)-C-(A)-(A)-U-(A)- --C-G-A U-G C-(A)-C-- C	11.8	2.1×10^5	8.3×10^5

helix II. The decline in complex stability between L25 and the different 5 S RNA mutants seems to be much more dependent on the stability of helix II as for L18. Note that an increase in the theoretical stability of helix II also leads to an increase in the binding constant between the 5 S RNA mutant and L25. These data strongly imply that our current view of the association sites between 5 S RNA and the ribosomal proteins L18 and L25 must be reconsidered and that some of the interpretations from limited enzymatic digestion and chemical modification studies have to be modified (see also [23]).

3.3. *Effects of 5 S RNA point mutations on tRNA binding*

Ribosomes containing mutated 5 S RNAs, with base changes at positions 41 (G to C), 66 (A to C) or 103 (U to G) were tested in their poly(U)-dependent Phe-tRNA binding capacity. The binding assay was performed as described [23] using ^{32}P-labeled 70 S ribosomes isolated from maxicells. Table 3 shows the relative tRNA and poly(U) binding activities of the mutants compared to wild-type ribosomes. The following conclusions can be drawn from the results presented in table 3: (i) All three mutants with base changes at either one of the positions 41, 66 or 103 show significantly reduced tRNA binding activities in our in vitro assay. (ii) While the mutants with base changes at positions 41 and 66 have a strongly reduced affinity for poly(U) the poly(U) affinity of the mutant at position 103 is in the same range as for the wild-type ribosomes.

The results have also been confirmed using non-radioactive ribosome preparations isolated from normal cells where a mixture of mutant and wild-type ribosomes is assayed. Since the cells containing plasmids with the mutated 5 S RNA genes do not show significant differences in their growth rates in YT medium we do not known how the deficiency in tRNA or mRNA binding of the mutant ribosomes is compensated in the cell. This is somewhat surprising because, according to our estimation, about 50% of the ribosomes contain mutated 5 S RNA.

Table 3

Affinity of ribosomes for poly(U) and Phe-tRNA

5 S RNA mutant	Poly(U)	Poly(U) + Phe-tRNA	Difference
Wild-type	11.9	17.9	6.0
pC 41	4.6	5.9	1.3
pC 66	7.2	7.0	–
pG 103	14.2	14.0	–

The numbers indicate the percentage of ribosomes retained on the affinity supports (see section 2). Values are averaged from 3 independent experiments

3.4. *Effects of base change mutations at the 3'-end of the 16 S RNA*

The 3'-end of the 16 S RNA has been proven in a number of studies to be of special functional importance for the translating ribosome. It plays an active role in initiation of translation (review [32]). A cross-link between base C_{1400} and a base of P-site-bound tRNA demonstrates the close spatial neighbourhood to the P-site [10]. A number of defined nucleotides within the 3'-end of the 16 S RNA show strong accessibility changes towards chemical modification in different functional states of the ribosome. The sequence position G_{1401} for instance is strongly protected from DMS methylation in ribosomes with bound tRNA as opposed to free ribosomes. Another example is G_{1416} which can be methylated by DMS in 70 S

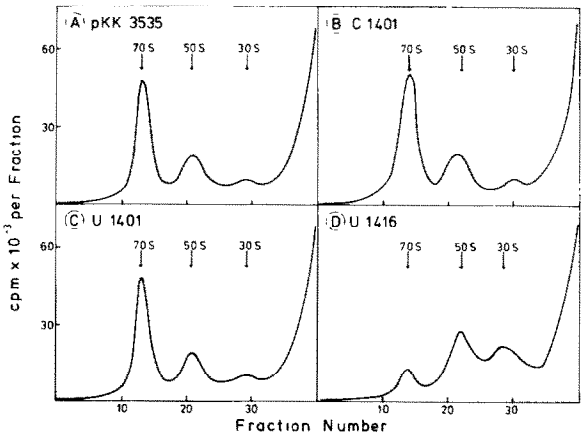

Fig.3. Sucrose gradient separation of ribosomes from cells containing different plasmids. Sedimentations were performed at 6 mM Mg^{2+}. Ribosomes were prepared after maxicell labeling and the radioactivity of the different fractions was monitored. (A) Wild-type ribosomes; in (B–D) the various base change mutations within the 16 S RNA gene are indicated.

ribosomes but seems to be unreactive in 30 S subunits [6,7]. Base change mutations were therefore constructed at these two sequence positions. The two double mutants C_{1399} to A, G_{1401} to C and C_{1399} to A, G_{1401} to U as well as the point mutant G_{1416} to U were tested for their functional influence on ribosomes containing these mutated RNAs. The growth rates in rich medium of all three mutants were not significantly altered when compared to cells containing the wild-type plasmid. All three mutant RNAs were processed correctly and incorporated into ribosomes. ^{32}P labeled ribosomes isolated from maxicells were consequently tested for their tRNA binding activity and subunit association behavior. Both maxicell ribosomes and the mixture of mutant and wild-type ribosomes isolated from normal cells were unaltered in their in vitro tRNA binding. (The same poly(U)-dependent Phe-tRNA binding assay as described for the 5 S RNA mutants was employed.)

When the three mutants were tested for impairments in subunit association by sedimentation in sucrose gradients in the presence of 6 mM Mg^{2+} the G_{1416} to U mutant gave a completely abnormal sedimentation profile (see fig.3). While the other mutants gave a 70 S peak comparable with the one obtained with wild-type ribosomes the U_{1416} mutant RNA containing ribosomes sedimented predominantly as 30 S subunits. One has to conclude that ribosomes with the U_{1416} mutation in their 16 S RNA do not form stable 70 S ribosomes under the sucrose gradient conditions. Comparison of the 16 S RNA from the small 70 S peak and the large 30 S peak from the gradient profiles of the U_{1416} mutant confirmed this notion. Whereas the 30 S peak consisted mainly of mutant U_{1416} ribosomal 16 S RNA the remaining 70 S peak was composed of wild-type 30 S ribosomes.

To investigate further whether the loss of 70 S stability was a direct effect of the altered 16 S RNA sequence or mediated by a disturbed protein interaction of the mutant ribosomes, we compared the protein composition of wild-type and mutant U_{1416} 30 S particles. Initial observations with mutant ribosomes containing an additional protein could not be confirmed, and a thorough investigation of the protein composition of the mutant and wild-type 30 S did not reveal a different protein pattern. The effect we observe must therefore be directly related to a structural alteration of 30 S particles containing the G to U base change at position 1416.

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